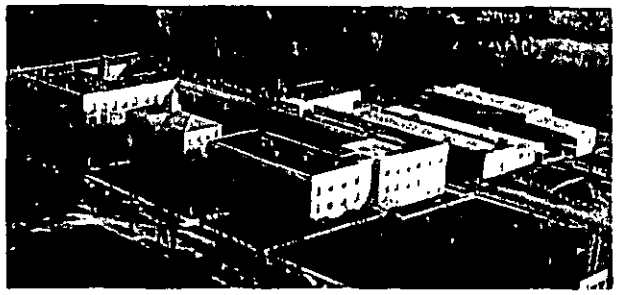

THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

THE GROWTH AND DIFFERENTIATION OF TISSUE ISOLATED FROM VARIOUS MEMBERS OF THE GENUS POPULUS

Project 2351

Report Three

A Progress Report

to

PIONEERING RESEARCH COMMITTEE

February 11, 1964

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TABLE OF CONTENTS

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THE GROWTH AND DIFFERENTIATION OF TISSUE ISOLATED FROM VARIOUS MEMBERS OF THE GENUS POPULUS

SUMMARY

1. A number of tissues were isolated from various members of the genus Populus. These tissues have been maintained on complex media containing coconut milk. Attempts to use a completely defined medium have been unsuccessful.

2. The secretion of materials from isolated aspen tissue was reported. Amylase was found in the medium while catalase was present in the tissue but not in the medium. Interesting patterns of bacterial growth were obtained when various aspen tissue cultures were inoculated with unidentified bacteria. The stimulation and inhibition of bacterial growth was observed.

3. Preliminary attempts to "control" the differentiation of isolated aspen tissue have been partially successful. Increased root initiation and growth was reported in the presence of 2 p.p.m. indoleacetic acid. Shoot initiation was not obtained.

INTRODUCTION

Recent developments in tissue culture techniques have led to a very diversified field of investigation. Isolated tissues have been utilized in studies involving the production of edible tissues, the growth of obligate parasites, the synthesis of drugs and other biologically active materials and the biochemical aspects of cellular growth and differentiation. This program has been designed to explore the possible uses of isolated tissue in studies dealing with the physiology of forest trees and to apply information available in the literature to problems involving the culture, growth, and differentiation of tissues isolated from various members of the genus Populus.

GENERAL RESULTS

A number of tissues from the genus Populus have been successfully isolated and are currently under culture (Appendix–Table V). These tissues have been maintained on agar medium (No. 23) containing major and minor elements, coconut milk, naphthaleneacetic acid, and sucrose (Appendix–Table VI). Attempts to isolate tissue from additional species have been unsuccessful.

The active growth of isolated aspen tissue requires the presence of coconut milk in the culture medium. A number of synthetic, completely defined media have been reported to support the growth and proliferation of certain tissues. Preliminary results (Appendix–Table VII) using a chemically defined medium (1) indicate that additional experimentation will be required in order to develop or select a suitable medium.

Isolated tissue, in general, is composed of thin-walled cells with scattered strands of conducting tissue (Fig. 1). This system, because of the relative ease of environment control, is well suited to studies involving the mechanism of differentiation.

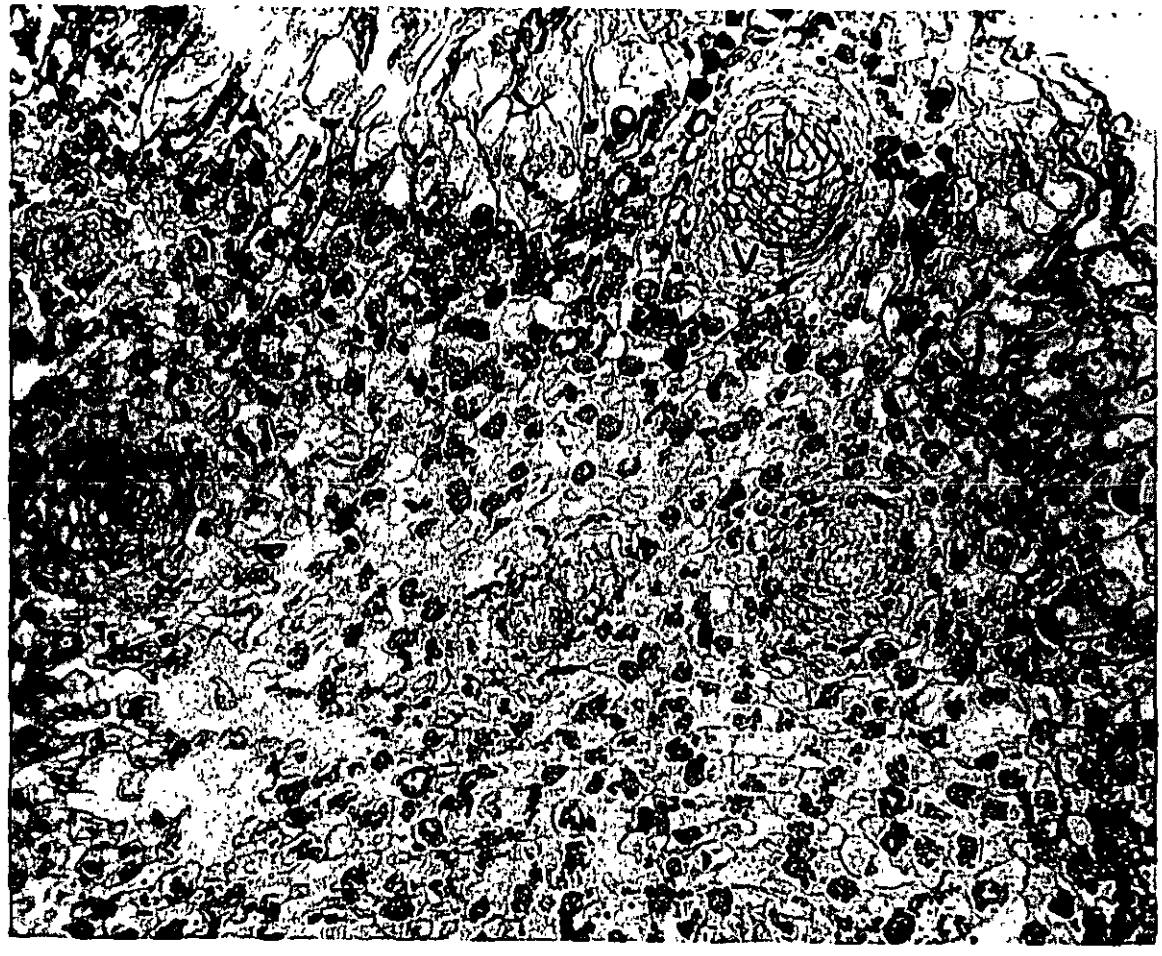

Figure 1. Microtome Section of Isolated Aspen Tissue
Showing Scattered Bundles of Vascular Tissue (VT)

SECRETION OF MATERIALS FROM ISOLATED TISSUES

ENZYME SECRETION

Amylase

Isolated aspen tissue (No. 16—see Appendix Table V) was cut into small pieces and placed on medium containing 1% soluble starch and allowed to grow for 3 weeks. Colorless zones were evident after the cultures were flooded with a few milliliters of starch indicator (Fig. 2). It was concluded that the growth of freshly cut isolated aspen tissue on a medium containing soluble starch resulted in the secretion of materials which digested starch in the medium surrounding the tissue. A few of the cultures were flooded with a suspension of Sarcina lutea or Pullularia pullulans and incubated until inhibitory zones were evident. The plates were removed from the incubator, flooded with starch indicator and observed. It was noted that the zone (colorless) of starch digestion did not coincide with the inhibitory zone. This indicated that the rate of diffusion or the time of production of the inhibitory substance was not similar to the material responsible for the digestion of starch in the medium.

The digestion of starch could be the result of simple diffusion of amylase from injured cells or it could be the result of the secretion of amylase from actively growing, uninjured cells. Freshly cut tissue (containing a number of cut and injured cells) was compared with tissue which had been growing for 3 weeks on medium containing soluble starch. The latter tissue was conditioned for 3 weeks to insure actively growing tissue, to reduce to a minimum the number of injured or dead cells and to allow for the diffusion of materials from the cells before the cut surfaces were healed. Tissue was placed on soluble starch medium after the conditioning period and allowed to grow for an additional 3 weeks. It

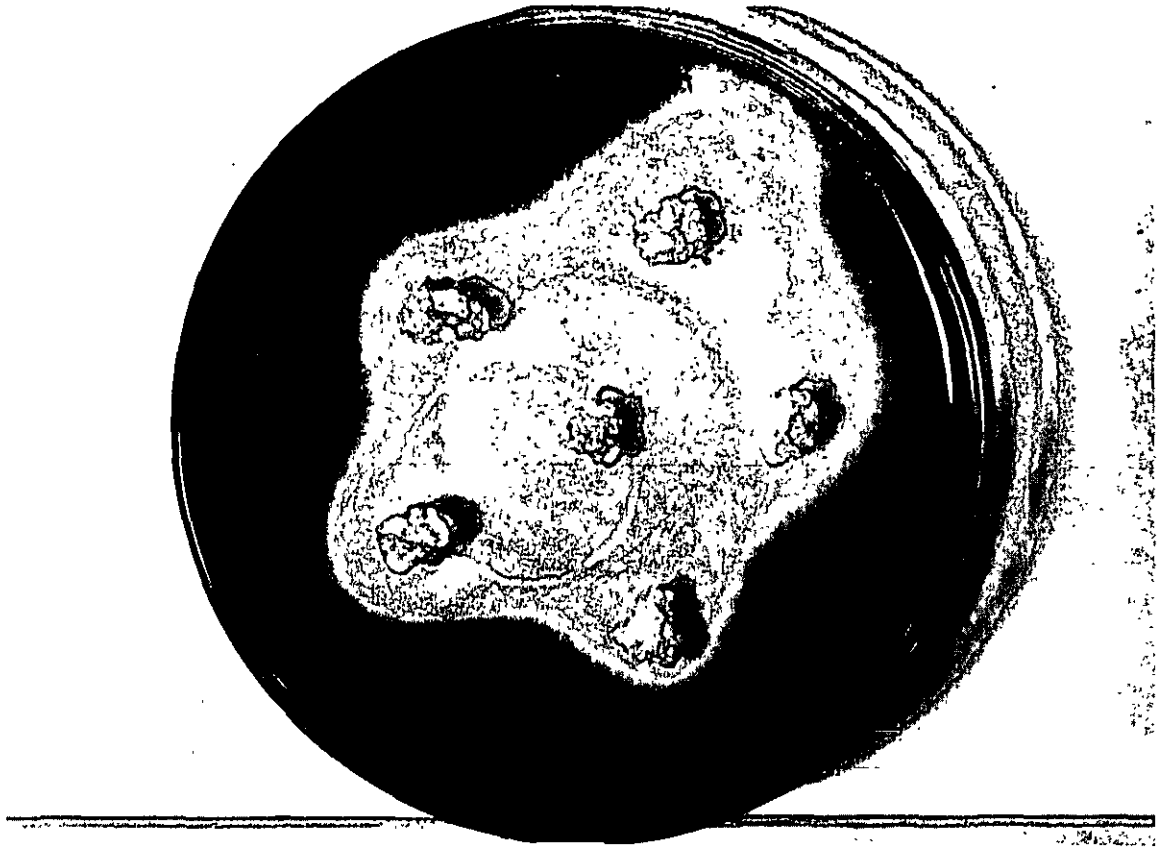

Figure 2. The Digestion of Starch (Clear Central Area) Resulting From the Growth of Isolated Aspen Tissue for a Period of 3 Weeks

was found that the growth of conditioned tissue on soluble starch medium also resulted in the digestion of starch in the medium surrounding the tissue. The growth of isolated quaking aspen tissue (TA-No. 16) resulted in zones with an average diameter of 40 mm. (Table I), while the growth of bigtooth aspen tissue (BT) resulted in zones with an average diameter of 34 mm. BT and TA tissue grew rapidly on a medium containing 2% sucrose (No. 23) and quite poorly in the absence of added sucrose (NS medium). The addition of soluble starch to NS medium (Table II) resulted in a slight increase in the total growth of the tissues, indicating that starch was utilized by the isolated tissues.

TABLE I

THE DIGESTION OF STARCH AS A RESULT OF THE GROWTH OF ISOLATED TISSUE ON SOLUBLE STARCH MEDIUM

Tissue[a]	Average Growth,[b] mg.	Average Increase,[c] %	Average Zone Diameter, mm.	pH Within Zone	pH Outside Zone
TA	195	447	40	5.9	5.8
BT	383	511	34	5.2	5.0

[a]TA - quaking aspen; BT - bigtooth aspen.
[b]Grown on medium containing 1% soluble starch.
[c]Based on fresh weight increase after 3 weeks.

TABLE II

THE GROWTH OF ISOLATED TISSUE ON SOLUBLE STARCH MEDIUM

Tissue[a]	Per Cent Increase[b] on Various Media[c] NS	2% SS	No. 23
TA	367	543	1267
BT	488	580	1055

[a]TA - quaking aspen; BT - bigtooth aspen.
[b]Based on fresh weight increase after 3 weeks.
[c]NS - No. 23 medium in the absence of added sucrose.
2% SS - No. 23 medium containing 2% soluble starch with no sucrose added.

Peroxidase-Catalase

Isolated tissue was cut into small pieces, placed on No. 23 medium and allowed to grow for approximately 3 weeks in the dark at 27-29°C. Actively growing tissue was selected at the end of the conditioning period and placed in plates containing fresh No. 23 medium. After the final growth period the tissue was removed and the plates were flooded with a 3% hydrogen peroxide solution followed by a solution of benzidine. The tissues were then placed in a small amount of hydrogen peroxide and observed. Immediate bubbling was noted. A positive test

for catalase was not obtained, however, when the medium was flooded with hydrogen peroxide. It was concluded that the tissue contained catalase and this enzyme either did not diffuse into the medium or was inactive in the medium. The addition of a few milliliters of benzidine to the tissue in hydrogen peroxide resulted in the appearance of a blue-violet color. This coloration was also evident when the medium was flooded with hydrogen peroxide and benzidine. In the case of the medium the development of color was not consistent. The color ranged from a faint blue-violet in the area surrounding the tissue to darker coloration immediately beneath the tissue. It was concluded that peroxidase was present in the tissues but inconclusive results were obtained with the diffusion of the enzyme into the medium. In some cases there was a definite secretion of peroxidase into the medium, while in other cases only a very small amount of enzyme was detected.

INHIBITORY MATERIALS

The production of antimicrobial substances by isolated aspen tissue (No. 16) has been observed and reported (2). It was found that the material or materials produced by the tissue and secreted out into the medium were active against a number of micro-organisms. The next phase of this program involved the isolation of tissue from various members of the genus Populus and the subsequent testing of these materials for the presence of antimicrobial materials.

Small pieces of isolated tissue were grown on agar medium (No. 23) for approximately three weeks in the dark at 27-29°C. The cultures were then flooded with a suspension of either Pullularia pullulans or Bacillus subtilis. Inhibitory zones were measured after additional incubation. Tissue isolated from triploid and diploid quaking aspen (P. tremuloides) and cottonwood (P. deltoides)

produced materials which resulted in the inhibition of both test organisms (Table III). Bacillus subtilis was inhibited by all of the isolated tissue. These results were consistent except in the case of bigtooth aspen (P. grandidentata) and P. davidiana tissue where some of the tissue did not produce distinct inhibitory zones even in the presence of adequate tissue growth. Distinct inhibitory zones were produced when cultures containing triploid or diploid quaking aspen, cottonwood, P. davidiana or P. canescens were inoculated with a suspension of Pullularia pullulans. The growth of tissue isolated from bigtooth aspen, white poplar (P. alba) did not result in the production of measurable inhibitory zones when the plates were flooded with Pullularia pullulans.

The apparent lack of inhibition in some cases may be due to a lower concentration of the inhibitory materials in the medium. A difference in the sensitivity of the two micro-organisms, coupled with concentration differences could result in the inhibition of one test organism while the other organism may grow at a normal rate. It was concluded that tissue isolated from various members of the genus Populus secretes materials which are similar in nature and result in the inhibition of selected micro-organisms.

STIMULATORY AND INHIBITORY MATERIALS

Small pieces of freshly cut isolated aspen tissue (No. 16) were placed on agar medium (No. 23) which had been seeded with an unknown contaminant. A few days incubation at 27-29°C. resulted in the production of distinct areas of increased bacterial growth (Fig. 3). Further experimentation indicated that the growth of this same contaminant was inhibited when isolated tissue was grown for 3 weeks prior to inoculation. The stimulation of growth could be the result of the simple diffusion of materials from the cut or injured cells on the surface

TABLE III

THE SECRETION OF ANTIMICROBIAL MATERIALS BY TISSUE ISOLATED FROM VARIOUS MEMBERS OF THE GENUS POPULUS

Tissue[a]	Bacillus subtilis			Pullularia pullulans				Remarks	
Genus Populus	Tissue Growth, mg. fresh wt. per piece of tissue	Aver. Diam. Zone, mm.	No. Pieces Tissue Tested	Aver. Tissue Growth, mg. fresh wt.	Aver. Diam. Zone, mm.	No. Pieces Tissue Tested	Growth Period	Inhibition Results	Tissue Growth
Triploid (No. 16) P. tremuloides	272	29	9	326	29	12	3 weeks	consistent	rapid
Diploid (No. 17) P. tremuloides	122	26	12	108	15	13	4 weeks	consistent	slow
P. grandidentata	185	12[b]	32	232	0[c]	15	3-4 weeks	inconsistent	moderate
P. deltoides	207	34	18	170	21	15	3-4 weeks	consistent	moderate
P. alba	187	17	15	182	0[c]	21	3 weeks	consistent	rapid
P. canescens	118	20	24	123	9[b]	18	3-4 weeks	inconsistent	moderate
P. davidiana	237	14[b]	22	201	2[b]	22	3-4 weeks	inconsistent	rapid

[a] Grown in the dark at 27-29°C. on basal medium containing 10% coconut milk and 0.5 p.p.m. napthaleneacetic acid.
[b] Some of the tissue examined did not produce a distinct inhibitory zone even in the presence of adequate tissue growth. Average includes cultures which did not produce inhibitory zones.
[c] Small areas of decreased growth of the organism were observed, but no distinct, measurable inhibitory zones.

Figure 3. The Stimulation of Bacterial Growth by Small Pieces of Freshly Cut Isolated Aspen Tissue (No. 16)

of the tissue or it could be due to the secretion of materials from actively growing uninjured cells. Preliminary results showed that the stimulation or inhibition of bacterial growth depended on the amount of tissue growth (length of growth period). An experiment was designed to clarify the secretion of these active materials from isolated aspen tissue.

Small pieces of isolated aspen tissue were cut, weighed, and placed on the surface of agar medium (No. 23). Each culture, containing 3 pieces of tissue, was incubated in the dark at 27-29°C. This procedure was repeated every 4 days in order to obtain tissue that had been grown on the medium for periods of 20, 16, 12, 8, and 4 days. At the end of the growth period the cultures were flooded with a suspension of the test organism (unidentified bacterium) in

nutrient broth. Freshly-cut tissue was also placed on the surface of No. 23 medium at the time of inoculation (0 days) with the test organism. The excess inoculum was removed and the cultures were incubated until sufficient growth of the test organism was evident.

The results of this experiment (Table IV, Fig. 4) showed the progressive steps between stimulation and inhibition. Initially a stimulatory zone was observed. This zone, which diffused out into the medium was followed by a very small zone of inhibition. This inhibitory zone was then followed by a small zone of stimulation and finally a zone of inhibition. The materials responsible for the stimulation of bacterial growth apparently were diluted, as a result of diffusion, and were not detectable after relatively large amounts of tissue growth. The larger pieces of tissue produced only inhibitory zones which increased in diameter with increased tissue growth.

The experiment was modified and repeated in order to investigate the possibility of the diffusion of materials from cut or injured cells. Small pieces of isolated tissue (No. 16) were cut, placed on agar medium (No. 23) and conditioned for a period of 4 days in the dark at 27-29°C. At the end of the conditioning period, cultures containing No. 23 medium and either freshly-cut or conditioned tissue were established and treated according to the previously described procedure. The tissue was conditioned in an attempt to insure actively growing tissue, to reduce to a minimum the number of injured or dead cells, and to allow for the diffusion of materials from the tissue before the cut surfaces were healed.

The results obtained, using either conditioned or freshly-cut tissue (Fig. 5), were identical to those obtained in the previous experiment which

TABLE IV

THE INHIBITION AND STIMULATION OF BACTERIAL GROWTH AS A RESULT OF MATERIALS SECRETED BY ISOLATED ASPEN TISSUE (NO. 16)

Growth Period of Tissue	Growth per Piece of Tissue,[a] mg.	Observations, bacterial growth[b]
20 days	164	All tissue produced an inhibitory zone. Average zone diameter - 20 mm.
16 days	93	All tissue produced an inhibitory zone. Average zone diameter - 13 mm.
12 days	67	All tissue produced an inhibitory zone. Average zone diameter - 10 mm.
8 days	31	1 piece of tissue produced an inhibitory zone. 5 pieces of tissue - outer diffuse area of stimulation followed by an area of inhibition, then an area of stimulation and a central (next to and below the tissue) region of inhibition.
4 days	22	6 zones as described for 8 days with outer stimulatory zones more distinct.
0 days	5[b]	6 stimulatory zones with very slight central inhibitory zones.

[a]Average of 2 cultures each containing 3 pieces of tissue.
[b]After incubation for 3 days.

Figure 4. The Influence of Freshly-Cut Isolated Aspen Tissue (No. 16) on the Growth of Bacteria. The Tissue Was Grown 0, 4, 8, 12, 16, and 20 Days Before Inoculation With the Micro-organism

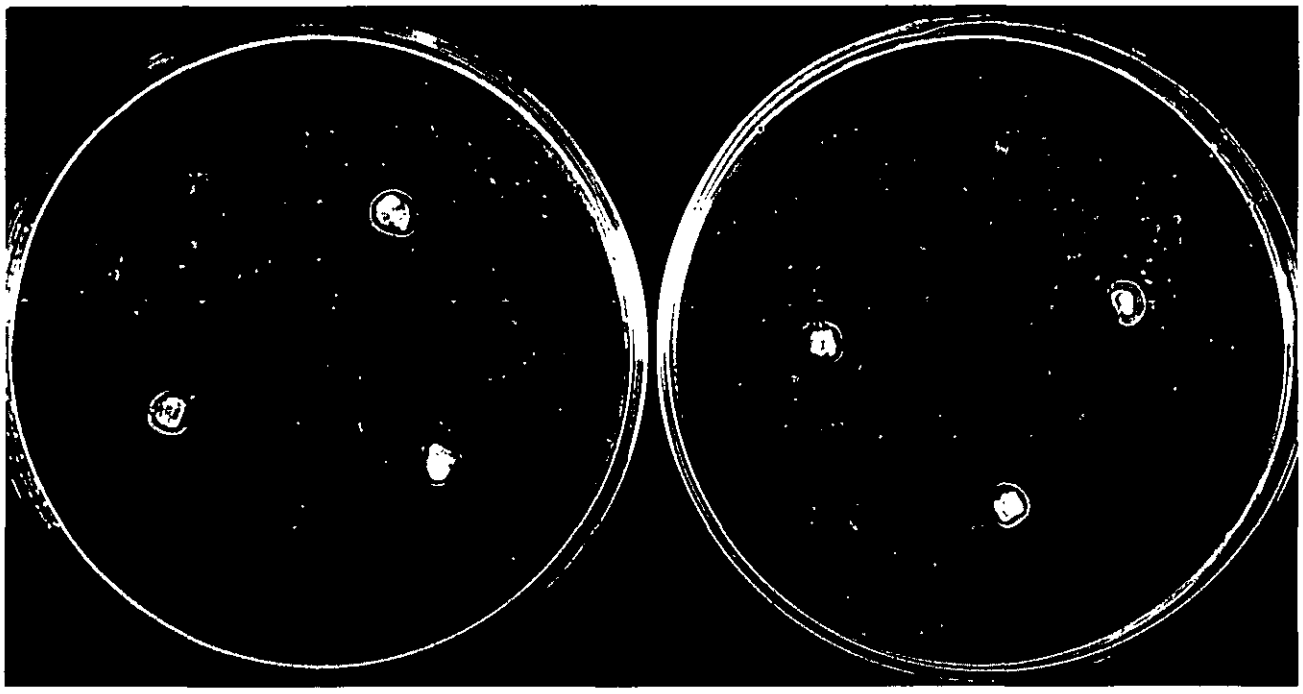

Figure 5. The Influence of Freshly-Cut and Conditioned Aspen Tissue (No. 16) on the Growth of Bacteria. The Tissue Was Grown 4 Days Before Inoculation with the Micro-organism

Left Plate Contains Freshly-Cut Tissue
Right Plate Contains Conditioned Tissue

utilized only freshly-cut tissue. The conclusions concerning the active materials produced in these experiments must be of a general nature because of the large number of possible explanations for the observed results. It can be stated, however, that isolated aspen tissue secretes into the medium materials which result in the production of distinct areas of stimulated or inhibited bacterial growth. These results are of interest because the demonstration of the inhibition or stimulation of bacterial growth does not require the extraction and concentration of materials from the isolated tissue. The materials which influence bacterial growth are present in concentrations which occur "naturally" in the culture medium.

DIFFERENTIATION

The work of Steward, et al. (3) has demonstrated the totipotency of single, isolated carrot cells. It is now possible to grow single cells and obtain root and shoot initiation by manipulating the chemical environment. These results suggest that "controlled" differentiation can be obtained using isolated cells from any plant species. The culture requirements vary between species, so it is reasonable to assume that the chemical environment which stimulates differentiation in one species may not influence differentiation in another species. Conditions which have been reported to stimulate the differentiation of isolated tissues will be utilized in this phase of the program. Additional treatments will be included in a systematic search for conditions which will stimulate the initiation of roots and shoots in isolated aspen tissue.

Lowenberg and Skoog (4) reported that the addition of a low level (approximately 0.1%) of citric acid promoted the budding of tobacco callus by counteracting the inhibitory effects of low levels of indoleacetic acid. The growth of aspen tissue (No. 16) on media containing citric acid resulted in a decrease in the rate of tissue growth and a decrease in the number of rooted sections (Appendix-Table VIII) when the concentration of citric acid was greater than 0.1%. Shoot initiation was not observed.

The nutrient level has also been reported to influence the amount of differentiation. Reinert (5) has found that increasing the phosphate concentration 5 or 6 times suppresses root formation and reinforces shoot formation. The growth of aspen tissue (No. 16) on media containing increased levels of phosphorus or potassium did not result in the production of shoots (Appendix-Table IX). Intermediate levels (3, 6, 9 times) of both elements increased the rate

of growth of the isolated tissue while the higher levels (12, 15, 18) decreased the rate of growth. Root initiation was stimulated by the higher levels of potassium but the growth of these roots was poor.

The influence of various growth regulators, especially kinetin and indoleacetic acid, on the differentiation of various tissues and intact plants has been the subject of numerous investigations. Skoog and Miller (6) found that the addition of low levels of kinetin to media containing 2 p.p.m. of indoleacetic acid resulted in a shift in the differentiation pattern. Stimulated root production was obtained at a concentration of 0.02 p.p.m. while a concentration of 0.5 p.p.m. resulted in the formation of shoots. An experiment was designed to explore the possibility of increased differentiation in the presence of various levels of kinetin and indoleacetic acid. Root development was obtained in the absence of added growth regulators (Appendix—Table X). The addition of indoleacetic acid, at a concentration of 2 p.p.m., was found to stimulate root production and root growth (Fig. 6). Shoot production was not observed.

It was concluded that root initiation, which occurs in a random nature on basal medium, may be stimulated by increasing the level of potassium or by the addition of indoleacetic acid. The most satisfactory root initiation and growth was obtained in the presence of 2 p.p.m. indoleacetic acid. The absence of shoot production in the various treatments suggests that additional factors or combinations of factors are required to stimulate the differentiation and growth of organized stems.

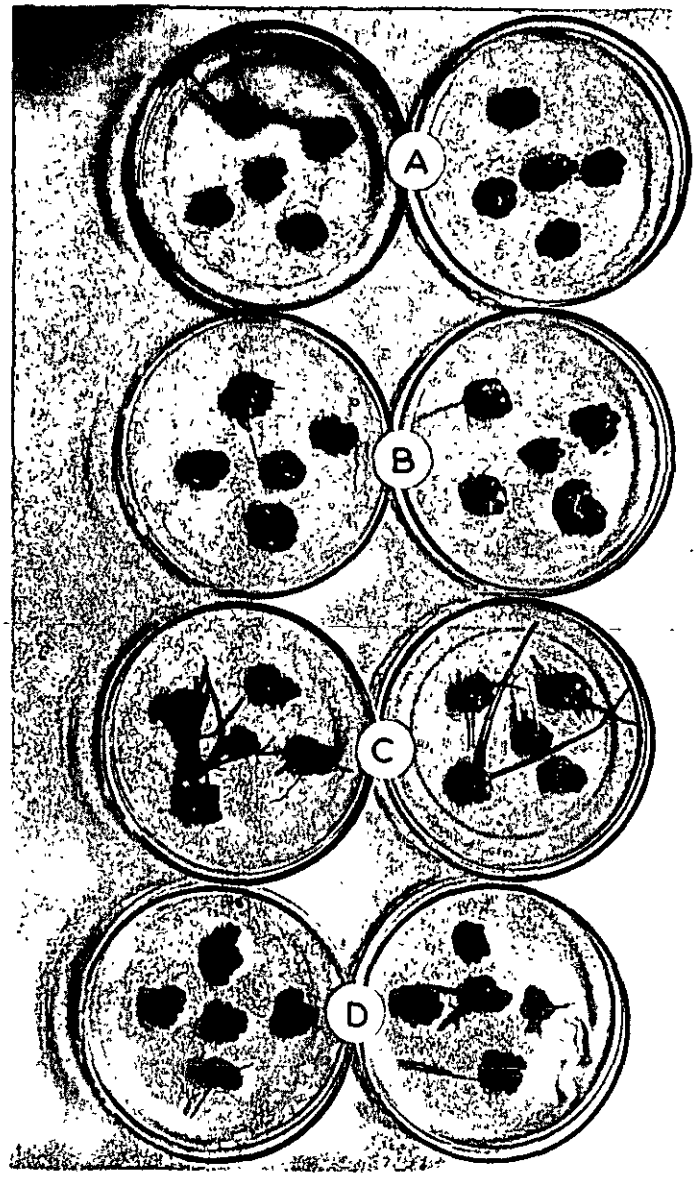

Figure 6. The in vitro Initiation and Growth of Roots as Influenced by Various Levels of Indoleacetic Acid

A - 6 p.p.m.
B - 4 p.p.m.
C - 2 p.p.m.
D - 0 p.p.m. (basal medium)

FUTURE PLANS

1. Isolate tissue from additional members of the genus Populus and from other genera in the Salicaceae.

2. Initiate experiments designed to determine the relationship between the rate of tree growth and the rate of callus growth in an isolated, carefully controlled system. The goal of this work will be to develop techniques for the evaluation of trees (in terms of growth rate) at a very early age.

3. Continue attempts to manipulate the chemical environment of the tissue to "control" differentiation. Additional growth regulators will be included.

4. Analyze samples of the various tissues in order to determine the concentrations of proteins, lignin, cellulose, and other constituents.

ACKNOWLEDGMENTS

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THE INSTITUTE OF PAPER CHEMISTRY

Martin C. Mathes

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APPENDIX

TABLE V

TISSUES ISOLATED AND MAINTAINED AT THE INSTITUTE OF PAPER CHEMISTRY. ALL TISSUES WERE ISOLATED FROM THE CAMBIAL AREA OF STEM SECTIONS

Parent Material: Species	Tree No.	Tissue Designation	Date of Isolation
Populus alba	A-2-59	Pa	8/28/63
Populus davidiana	Da-4, S1	Dav	8/28/63
Populus canescens	Ca-2	Can	9/16/63
Populus tremuloides (triploid)	T-2-56	Ta (No. 16)	12/26/61
Populus tremuloides (diploid)	XT-0-43-56 (No. 37)	Da (No. 17)	5/14/62
Populus grandidentata	G-7-62	Bt	1/27/63
Populus deltoides	XD-0-45-62	Cw	1/8/63

TABLE VI

CHEMICAL COMPONENTS OF NO. 23 MEDIUM[a]

Chemical	Weight, g./liter	Chemical	Weight, mg./liter
Na_2SO_4	0.200	$NaH_2PO_4 \cdot H_2O$	16.0
$Ca(NO_3)_2 \cdot 4H_2O$	0.200	Ferric citrate	10.0
$MgSO_4 \cdot 7H_2O$	0.360	$MnSO_4$	3.0
KNO_3	0.080	$ZnSO_4 \cdot 7H_2O$	0.5
KCl	0.066	H_3BO_3	0.5
Sucrose	20.0	$CuSO_4 \cdot 5H_2O$	0.025
Agar	8.0	$Na_2MoO_4 \cdot 2H_2O$	0.025
Coconut milk[b]	10%	Glycine	0.3
		Thiamine·HCl	0.01
		Naphthaleneacetic acid	0.5

[a] No. 12 - in the absence of naphthaleneacetic acid.
[b] Heated to 60°C., cooled and filtered.

TABLE VII

THE GROWTH OF ISOLATED ASPEN TISSUE IN THE ABSENCE OF COCONUT MILK

Medium	Increase, % 3 Weeks	6 Weeks	9 Weeks
No. 23 with no added coconut milk	586	1008	1316
Synthetic	851	--[a]	--
No. 23	1110	3102	5701

[a]Poor growth was obtained after 3 weeks.

TABLE VIII

THE INFLUENCE OF CITRIC ACID ON THE GROWTH AND DIFFERENTIATION OF ISOLATED ASPEN TISSUE (NO. 16)

Medium[a] Citric Acid, %	Increase[b], % 4 Weeks (% Control)	Number of Rooted Sections[d] 4 Weeks	8 Weeks[c]
0 (Control)	100	0	5
0.1	118	0	1
0.2	42	0	1
0.4	8	1	1
0.8	.05	0	0

[a]Autoclaved citric acid added to No. 23 medium.
[b]Based on fresh weight.
[c]4 Weeks growth of citric acid medium + 4 weeks growth of No. 23 medium.
[d]Each treatment contained 10 sections.

TABLE IX

THE INFLUENCE OF VARIOUS LEVELS OF POTASSIUM AND PHOSPHORUS ON THE GROWTH AND DIFFERENTIATION OF ISOLATED ASPEN TISSUE (NO. 16)

Treatment[a]	3 Weeks Growth (% Control)	No. Rooted Sect.[b] 4 Weeks	8 Weeks
1XP (Control)	100	1	2
3XP	153	0	3
6XP	135	0	2
9XP	119	0	1
12XP	67	1	1
15XP	96	0	2
18XP	82	0	0
1XK (Control)	100	1	2
3XK	133	0	0
6XK	134	0	2
9XK	143	0	1
12XK	42	6	9
15XK	94	1	8
18XK	64	3	11

[a]Numbers indicate the number of times the concentration of either phosphorus or potassium was increased. Control was No. 23 medium.
[b]Each treatment contained a total of 15 pieces of tissue.

TABLE X

THE INFLUENCE OF VARIOUS LEVELS AND COMBINATIONS OF INDOLEACETIC ACID AND KINETIN ON THE DIFFERENTIATION OF ISOLATED ASPEN TISSUE (NO. 16)

Medium[b], p.p.m.		No. Rooted Sections[a]	
Kinetin	Indoleacetic Acid	4 Weeks	8 Weeks[c]
0	0	2	4
0	2	3	8
0	4	0	6
0	6	0	5
.5	0	3	6
.5	2	2	5
.5	4	0	3
.5	6	4	5
2	0	0	1
2	2	0	5
2	4	0	3
2	6	0	4
5	0	0	2
5	2	0	1
5	4	0	1
5	6	0	1

[a] Each treatment contained 10 pieces of tissue.
[b] No. 12 medium (in the absence of naphthaleneacetic acid).
[c] Transferred after 1 month to No. 23 medium and allowed to grow for an additional month.